Elsevier Editorial System(tm) for Animal Reproduction Science

Manuscript Draft

Manuscript Number: ANIREP-D-16-6441R2

Title: The sheep conceptus modulates proteome profiles in caruncular endometrium during early pregnancy

Article Type: Research paper

Keywords: sheep, endometrium, proteome, gravid and non-gravid uterine horns, early pregnancy

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Abstract: The stage-specific expression of functional proteins within the endometrium, and their regulation by conceptus-derived signals, are crucial for conceptus development and successful establishment of pregnancy. Accurate knowledge of endometrium-conceptus interactions is key for the development of effective strategies to improve conceptus implantation rates both following natural conception and/or assisted reproductive technologies. The unilateral pregnant ewe provides a powerful experimental model for the study of endometrial function in the presence or absence of conceptuses during the peri-implantation period. Two-dimensional gel electrophoresis and mass spectrometry-based proteomics were used to compare and identify differentially expressed proteins in caruncular endometrium collected from the gravid uterine horns and the non-gravid uterine horns at the time of conceptus attachment (day 16 of pregnancy) and early post-implantation period (day 20 of pregnancy). Fifty seven protein spots were up-regulated in the gravid horn at day 16 of pregnancy and twenty seven protein spots were up-regulated in the gravid horn at day 20 of pregnancy. Sixteen proteins with different functions such as protein metabolism, cholesterol and ion transport and cell adhesion were identified. In conclusion, the use of the unilaterally pregnant ewe model provides evidence that the early implantation and post-implanting conceptus-derived signals up-regulate caruncle endometrial proteins, including carbonic anhydrase 2 (CA-II) and apolipoprotein A-1 (APOA1) and down-regulate caruncle endometrial proteins, including adenosylhomocysteinase (AHCY) and heat shock 60kDa protein 1 (HSP60). These regulated proteins are likely involved in providing a suitable intra-uterine environment required for conceptus attachment, implantation, early post-implantation development and the successful establishment of pregnancy in sheep.

Anim Reprod Sci: Ms # ANIREP-D-6441

Title: The sheep conceptus modulates proteome profiles in caruncular endometrium during early pregnancy.

Authors: Arianmanesh M, Fowler PA and Al-Gubory KH.

Point-by-point response to the reviewer comments.

Reviewer 1: The paper has been improved by the revisions made.

Authors' comment

We thank the reviewer for this positive feedback. The reviewer understood the main goal of our manuscript and the implications of our method.

The fact that several of the spots in Table 2 are associated with more than one protein ID is still not explained anywhere.

We apologies for this oversight and have addressed this in Table 2 and its footnotes with respect to the 3 protein spots showing a secondary identification.

Line 222 should be associated with

Line 264 should be involved

Line 286 should be increased

Line 287 should be evidence

Authors' comment

We apologize for these errors, and we have corrected the text as suggested.

Reviewer 2:

Reviewer's response to revised manuscript.

In their response to reviewer's comments, the authors admit that in their study, data on PGE2 and its receptor are sadly lacking. The authors even cite the 2011 reference by Dorniak et al (Biol Reprod 84:1127) which clearly shows the critical role of PGE2 the early establishment of early pregnancy in sheep but they do not include this reference in their revised Ms. A more recent comprehensive review confirms the critical role of PGE2 and its receptor in the early establishment of pregnancy in ruminants (Arosh et al, 2016 J Dairy Sci 99:5926).

So the bottom line is that this article is incomplete because it does not include data on PGE2 and its receptor in the two models that they employed. The study would be greatly improved by including two additional controls with non-pregnant ligated uterine horns in addition to the ovariectomized non-pregnant ligated uterine horn that they originally employed. First, in addition to their original control of the ovariectomized non-pregnant ligated uterine horn, they should also include the ligated non-pregnant uterine horn but with its ovary included. Second, they should also include animals with a ligated uterine horn but this time with a corpus luteum in the ovary. This is important because it is established in sheep that progesterone from the corpus luteum reaches its adjacent uterine horn locally at a much higher concentration than reaches it via the systemic circulation.

Such an improved experimental design together with the inclusion of measurement of PGE2 and its receptor would make an important contribution to our knowledge of the early establishment of pregnancy in ruminants

Authors' comment

We thank the reviewer for his/her in-depth analysis, useful comments, valuable time and useful contribution.

The reviewer asked to perform significant amount of experiments by using different sheep models together with the inclusion of measurement of PGE2 and its receptor to answer a specific point, which falls outside the scope of this study and is far from the rational of our study clearly stated in the introduction by the following paragraph (page 3, lines 53-57):

"Although a multitude of molecular pathways involved in extraembryonic membrane-endometrium crosstalk during conceptus implantation and post-implantation periods have been identified through studies of gene expression, a comprehensive understanding of changes in many endometrium proteins expressed in the presence of conceptuses is currently lacking. "

We believe that our paper, whose conclusions are not in doubt, is complete as it is, and as often is the case, more focused researches are still needed. But the paper is designed to answer an important outstanding question, and it does so. These requested experiments would not change the conclusion of the paper.

The unilateral pregnant sheep model used in the present study provides a new understanding about the role of conceptus-derived signals in the regulation of functional endometrial proteins involved in iron transport and homeostasis, hydrolysis, protein chaperoning and degradation, amino acid metabolism, cholesterol transport and cell adhesion.

We strongly think that the findings reported in our study establish a new reference database and will open the avenue for future follow-up mechanistic studies toward understanding the role, if any, of known (INFtau and/or PGE2) and unknown concepts-derived factors in the regulation of caruncle endometrial proteins, including carbonic anhydrase 2, apolipoprotein A-1 (APOA1), adenosylhomocysteinase and heat shock 60kDa protein 1.

Directions for future research are now open such that the present study provides a stimulus for further research.

Dear Editor,

We acknowledge with thanks receipt of your e-mail of 21 September 2016 concerning our manuscript:

Ms. No. ANIREP-D-16-6441R1
The sheep conceptus modulates proteome profiles in caruncular endometrium during early pregnancy

We are pleased to inform you that the manuscript was revised as requested.

Thank you very much for giving us the opportunity to publish our study in Animal Reproduction Science.

Yours sincerely,

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Highlights

1. The unilateral pregnant ewes were employed to investigate proteome changes during the peri-implantation period.

2. Conceptus-derived signals regulate multiple functional proteins in caruncular endometrium.

3. These proteins likely provide a suitable environment required for conceptus implantation and development.

The sheep conceptus modulates proteome profiles in caruncular endometrium during early pregnancy

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Abbreviated title: Conceptus control of endometrium protein expression

Keywords: sheep, endometrium, proteome, gravid and non-gravid uterine horns, early pregnancy

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Abstract

The stage-specific expression of functional proteins within the endometrium, and their regulation by conceptus-derived signals, are crucial for conceptus development and successful establishment of pregnancy. Accurate knowledge of endometrium-conceptus interactions is key for the development of effective strategies to improve conceptus implantation rates both following natural conception and/or assisted reproductive technologies. The unilateral pregnant ewe provides a powerful experimental model for the study of endometrial function in the presence or absence of conceptuses during the peri-implantation period. Two-dimensional gel electrophoresis and mass spectrometry-based proteomics were used to compare and identify differentially expressed proteins in caruncular endometrium collected from the gravid uterine horns and the non-gravid uterine horns at the time of conceptus attachment (day 16 of pregnancy) and early post-implantation period (day 20 of pregnancy). Fifty seven protein spots were up-regulated in the gravid horn at day 16 of pregnancy and twenty seven protein spots were up-regulated in the gravid horn at day 20 of pregnancy. Sixteen proteins with different functions such as protein metabolism, cholesterol and ion transport and cell adhesion were identified. In conclusion, the use of the unilaterally pregnant ewe model provides evidence that the early implantation and post-implanting conceptus-derived signals up-regulate caruncle endometrial proteins, including carbonic anhydrase 2 (CA-II) and apolipoprotein A-1 (APOA1) and down-regulate caruncle endometrial proteins, including adenosylhomocysteinase (AHCY) and heat shock 60kDa protein 1 (HSP60). These regulated proteins are likely involved in providing a suitable intra-uterine environment required for conceptus attachment, implantation, early post-implantation development and the successful establishment of pregnancy in sheep.

Introduction

In sheep, goats and cattle, successful conceptus (embryo and associated extraembryonic membranes) implantation relies on elaborate cellular, biochemical and molecular cross-talk between the extraembryonic membranes and receptive uterine endometrial tissues that ensures corpus luteum (CL) progesterone production and optimal post-implantation conceptus development and survival (Paria et al., 2001; Imakawa et al., 2004). During early pregnancy, a high rate of embryonic morality occurs due to abnormal conceptus signalling (Goff, 2002; Dixon et al., 2007; Diskin and Morris, 2008). Although a multitude of molecular pathways involved in extraembryonic membrane-endometrium crosstalk during conceptus implantation and post-implantation periods have been identified through studies of gene expression, a comprehensive understanding of changes in many endometrium proteins expressed in the presence of conceptuses is currently lacking.

Our previous studies provided original evidence that several endometrial proteins with different functions, including protein synthesis and degradation, antioxidant defence, cell structural integrity, adhesion and signal transduction, play important roles in the establishment of early pregnancy in sheep (Al-Gubory et al., 2014). Of particular interest were proteins that were highly expressed in response to the presence of conceptuses at attachment and early post-implantation periods and, using our unilaterally pregnant ewe model, we demonstrated that the early implantation and post-implanting conceptus-derived signals up-regulate the expression of cytoplasmic tryptophanyl tRNA synthetase and the mitochondrial superoxide dismutase (Al-Gubory et al., 2014). The former is a key catalytic enzyme for the first step reaction in protein synthesis (Sallafranque et al., 1986) and the latter the first antioxidant defence enzyme against reactive oxygen species-induced mitochondrial oxidative damage (Orrenius et al., 2007), in sheep caruncular endometrium (Al-Gubory et al., 2015). In sheep, the endometrium caruncles (CAR) are highly vascularized stromal protuberances covered by a simple luminal epithelium. CAR areas are specialized sites of attachment of the outer covering extraembryonic membrane, the trophectoderm, and are privileged

endometrial tissues for conceptus-uterine communication. Our hypothesis is that the early developing sheep conceptus modulates protein expression profiles in CAR endometrium during early pregnancy.

The unilateral pregnant sheep model enables changes in the expression of endometrial proteins in the presence or absence of conceptuses to be studied, providing a powerful model for the investigation of proteome changes during the peri-implantation period (Al-Gubory et al., 2015). The benefit of this model is that both uterine horns are exposed to similar concentrations of circulating hormones such as progesterone but only the gravid horn is under the direct action of local signalling molecules produced by the conceptus. In the present study, the unilateral pregnant ewes with functional ovaries were therefore employed to test our hypothesis. Two-dimensional gel electrophoresis (2DE) based proteomics (Fowler et al., 2007; Arianmanesh et al., 2011; Al-Gubory et al., 2014) was used to characterize specific alterations in the proteome of CAR endometrial tissues collected from the gravid uterine horns (GH) and the non-gravid uterine horns (NG) at the time of conceptus attachment (day 16 of pregnancy) and early post-implantation period (day 20 of pregnancy).

Materials and Methods

Experimental animals

All procedures relating to care and use of animals were approved by the French Ministry of Agriculture according to the French regulation for animal experimentation (authorization no° 78-34). Ewes of the Préalpes-du-Sud breed (18 months of age) were used in this study. Unilaterally pregnant ewes were prepared surgically as described previously (Payne and Lamming, 1994; Lamming et al., 1995). Briefly, ewes were initially anesthetized with a mixture of pentobarbital (Sanofi, Paris, France) and thiopentone (Abbott, Aubervilliers, France). After endotracheal intubation, general anaesthesia was maintained by constant inhalation of a mixture of oxygen and halothane. Reproductive organs were exposed via midventral laparotomy and one ovary was removed. One uterine horn was ligated close to the uterine bifurcation so that after mating the

conceptus is confined to the non-ligated pregnant horn. Three weeks after surgery, the ewes were treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France) to synchronize oestrous. Ewes were mated twice with fertile rams of the same breed, at an interval of 12 h during the synchronized oestrus. The ewes were housed under conditions of natural day-length and temperature and had free access to mineral licks and water.

Endometrial tissue collection

The ewes were slaughtered at a local abattoir in accordance with protocols approved by the local institutional animal use committee at the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France). Pregnant ewes were randomly allocated for slaughter at two specific stages of early pregnancy corresponding to the initial conceptus attachment to CAR areas (day 16, n=4 ewes) and the early conceptus post-implantation period (day 20, n=4 ewes). The stages of pregnancy were confirmed by the presence and the morphology of the conceptus in uterine flushings. Immediately after slaughter of the ewes, the reproductive tracts were collected, placed on crushed ice and transported to the laboratory. Endometrial CAR areas were collected from the entire GH (ipsilateral uterine horn) and NG (contralateral uterine horn) of each ewe, snap-frozen in liquid nitrogen and stored at -80 °C until processed for 2DE gel electrophoresis and Western blot.

Protein extraction and quantification for electrophoretic analysis

CAR from the gravid horns on days 16 (GH16) and 20 (GH20), non-gravid horns on days 16 (NG16) and 20 (NG20) were processed separately for 1DE and 2DE gel electrophoresis as described previously (Fowler et al., 2007). Briefly, tissues were combined with 5 ml lysis buffer/1 mg wet weight of tissue. The lysis buffer (0.01 M Tris–HCl, pH 7.4) contained 1 mM EDTA, 8 M urea, 0.05 M dithiothreitol, 10% (v/v) glycerol 5% (v/v), NP40, 6% (w/v), pH 3–10, resolyte (Merck Eurolab Ltd, Poole, Dorset, UK) and protease inhibitor cocktail (Roche Diagnostics). The tissues were disrupted using a Tissue Lyser (Qiagen Ltd) for 4 min at 30 Hz. Insoluble materials were removed from the lysates by centrifugation (50,000 g at 4°C) for 30 min. The protein content

of the final supernatant had been determined by RC-DC assay (Bio-Rad Laboratories Ltd). The protein extracts were stored at -80°C until required for further analysis.

Two dimensional gel electrophoresis (2DE) analysis

Equal amounts of protein from CAR of each ewe in each group were combined to make 4 protein pools (800 μg protein in each pool): gravid horn on days 16 (GH16) and 20 (GH20), non-gravid horn on days 16 (NG16) and 20 (NG20). 2DE was performed as described (Cash et al., 2003). As a first dimension separation, 70 μg of total protein from each pool was loaded onto 7 cm immobiline™ DryStrip non-linear pH gradient (IPG) strips of pH 3-10 (GE Healthcare, UK). The second dimension was carried out using 13 cm NUPAGE® Novex 4-12%, Bis-Tris Zoom® gels (Invitrogen Ltd, Paisley, UK). Quadruplicate 2DE gels were prepared for each of the 4 groups (representative gel is shown in Figure 1). Proteins were visualized using Colloidal CBB G-250 and scanned using an ImageScanner™ III (GE Healthcare). Protein spot profiles were analysed using Progenesis SameSpots version 3 software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) as described (Arianmanesh et al., 2011). Briefly, reference gel was selected and the other gels were aligned to be closely matched to this reference gel. Background was subtracted individually from each gel and spot volumes were normalised relative to total spot volume individually for each gel. Ultimately, 15 were selected for identification by LC-MS/MS on the basis of significance (log-normalised spot volumes had to differ between two groups at the level of $P<0.05$ by ANOVA and post-hoc testing), spot volume (a difference of a $\geq$1.25-fold increase or decrease between two groups), concentrating on the most abundant proteins with the most stable expression across the 4 replicate gels for each group.

Mass spectrometry

To identify proteins, 15 selected spots were excised from stained gels and subjected to in-gel trypsin digestion as described previously (Uwins et al. 2006). The peptide fragment mass spectra

were acquired on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer operated in the reflection delayed extraction mode. Tryptic peptides from the MS/MS spectra were applied to search the NCBI (National Centre for Biotechnology Information) database with the MASCOT program (http://www.matrixscience.com). Search parameters for the programme included maximum allowed error of peptide mass 250 ppm, cysteine as S-carbamidomethyl-derivative and oxidation of methionine were allowed.

Statistical Analysis

Normality of data was tested with the Shapiro-Wilk test. Normally distributed data were subjected to one- and two-way ANOVA and Bonferroni post-hoc test using SPSS 17.0 software to assess significance of differences. Statistical comparisons between specific groups were carried out by student's t-test. Differences were considered significant at $P<0.05$.

Results

Overall, 998 protein spots were included (on the basis of clear, reproducible expression and absence of noise in all four gels for each group) for analysis from a total of 1482 distinct protein spots detected by automatic detection with Progenesis SameSpots Software. The number of spots showing statistically significant differences in normalized spot volumes between groups is shown in Table 1.

Comparison between the GH and the NG uterine horns at day 16 of pregnancy revealed that 47 (3%) of protein spots were significantly changed ($P<0.05$). Among these, 35 normalized spot volumes were up-regulated and 12 normalized spot volumes were down-regulated (Table 1). Comparison between the GH and the NG uterine horns at day 20 of pregnancy revealed that 27 (2%) of protein spots were significantly changed ($P<0.05$). 25 of these normalized spot volumes were up-regulated and 2 normalized spot volumes were down-regulated (Table 1). In GH uterine horns, 48 (3%) of protein spots were significantly changed ($P<0.05$) between days 16 and 20 of

pregnancy. In this category, 17 normalized spot volumes were up-regulated and 31 normalized spot volumes were down-regulated (Table 1). In NG uterine horns, 48 (3%) of protein spots were significantly changed ($P<0.05$) between days 16 and 20 of pregnancy. Among these, 30 normalized spot volumes were up-regulated and 18 normalized spot volumes were down-regulated (Table 1).

The proteins spots in GH and NG uterine horns exhibiting significant differences in expression at implantation day and early post-implantation period and identified are shown in Table 2. Adenosylhomocysteinase (AHCY, Figure 2A) increased ($P<0.05$) in NG uterine horns compared to GH uterine horns at both days 16 and 20 of pregnancy. (Table 2). Carbonic anhydrase 2 (CA-II, Figure 2B), increased ($P<0.05$) in GH uterine horns compared to NG uterine horns at both days 16 and 20 of pregnancy (Table 2). Heat shock 60 kDa protein 1 (HSP60, Figure 2C), increased ($P<0.05$) in NG uterine horns compared to GH uterine horns at day 20 of pregnancy. (Table 2). In GH uterine horns, HSP60 decreased (Figure 2C, $P<0.05$) at day 20 when compared with day 16 of pregnancy (Table 2).

In GH and NG uterine horns, proteasome activator subunit 2 (PA28beta/PSME2, Figure 3A) increased ($P<0.05$) at day 20 when compared with day 16 of pregnancy (Table 2). In GH uterine horns, apolipoprotein A-1 (APOA1, Figure 3B) increased ($P<0.05$) at day 20 when compared with day 16 of pregnancy (Table 2). In GH and NG uterine horns, transferrin (TF, Figure 3C) decreased ($P<0.05$) at day 20 when compared with the day 16 of pregnancy (Table 2). In GH uterine horns, galectin 15 (LGALS15, Figure 3D) decreased ($P<0.05$) at day 20 when compared with day 16 of pregnancy (Table 2).

Discussion

The establishment of pregnancy requires correctly timed, exquisitely coordinated, adaptive, responses of the uterine endometrium to the presence of conceptuses. Some of these responses will be via modification of the expression of functional proteins during early pregnancy. The proteomic profile of sheep CAR endometrium reported here provided a new understanding about the role of

conceptus-derived signals in the regulation of a substantial number of functional proteins involved in iron transport and homeostasis, hydrolysis, protein chaperoning and degradation, amino acid metabolism, cholesterol transport and cell adhesion.

Conceptus-derived factors reduce the expression of AHCY protein as evidenced by the down-regulation of this protein in endometrial caruncular tissues of the gravid uterine horns compared with the non-gravid uterine horns at both implantation and post-implantation periods (present study). AHCY catalyzes the breakdown of S-adenosylhomocysteine (AdoHcy) to adenosine (Ado) and L-homocysteine (Hcy) (Turner et al., 2000). It is imporatnt to note that hyperhomocysteinemia (HHcy) exerts adverse effects through the induction of inflammation pathways, including endothelial monocyte adhesion and infiltration (Wang et al., 2002), oxidative stress, activation of pro-inflammatory factors and endothelial dysfunction (Lawrence de Koning et al. 2003). Hcy activates NADPH oxidase and increases reactive oxygen species in human umbilical vein endothelial cells (Dong et al., 2005). Elevated level of Hcy within organs and tissues is therefore a potentially pathophysiological risk factor for uterine endothelial function via an enhancement of oxidative stress and inflammation. Maternal Hhcy shoud be associated with placental abruption and spontaneous abortion (Ray and Laskin, 1999). Increased Hcy levels and oxidative stress represent a risk factor for the establishment and maintenance of pregnancy (Micle et al. 2012). Therefore, the conceptus must hold Hcy in check within CAR endometrium at conceptus attachment (Day 16) and early post-implantation period (Day 20) of pregnancy through down-regulation of AHCY protein expression (present study). We suggest that the conceptus-derived factors exert local effects within the endometrium to counteract peri-implantation oxidative stress through the control of Hcy production and thereby support the establishment of pregnancy.

Upregulation of CA-II protein expression by conceptus-derived signals observed here on the day of conceptus implantation and during early post-implantation period had not been reported previously in endometrium of any mammalian species. Identification of these signaling molecules is essential in our understanding of the molecular mechanisms that should be involved in the establishment of

pregnancy. CAII catalyzes the reversible hydration of carbon dioxide to bicarbonate and plays an important role in acid-base homeostasis within tissues of biological systems (Khalifah, 1971). These reactions are requisite for cancer development, invasion and progression. Interestingly, CA II is highly expressed in tumours of different organs, including brain (Parkkila et al., 1995a), pancreas (Parkkila et al., 1995b) and kidney (Parkkila et al., 2000), where it favourably induces an environment necessary for the growth and spread of the tumour by changing acidity of the extracellular medium surrounding cancer cells. In the neonatal mouse uterus, where members of the CA family are expressed (Hu et al., 2004), CAII mRNAs were localized in epithelial and stromal cells of the endometrium suggesting a functional role for CAII in endometrial gland development during postnatal uterine development (Hu and Spencer, 2005). The expression of CA II in the bovine (Nishita et al., 1990) and human (Aliakbar et al., 1990; Muhlhauser et al., 1994) placentas supports the suggestion that this enzyme is required for endometrial tissue remodelling. Endometrium structural remodelling in ruminants, including sheep, plays crucial role in implantation, placentation and conceptus nutrition (Igwebuike, 2009). On the day of conceptus attachment (day 16 of pregnancy), there is close contact between trophoblast, the extra-embryonic membrane of the conceptus, and the epithelium overlying CAR endometrium, over raised areas of the endometrium, to allow implantation and early placental development. The high level of CA-II protein expression in CAR endometrium of the gravid uterine horns (present study) likely suggests an important role for this regulated protein in promoting trophoblast attachment, invasion and fusion with endometrial epithelium and/or remodelling the endometrium for successful early conceptus implantation and, consequently, formation of the maternal-fetal interface during placental development.

HSP60 has multiple functions in the normal cells, including inter-organelle transport, inhibition of aggregation of denatured polypeptides, antigen presentation, pro-apoptotic activity (Yu et al., 2012) and promotion of the proper folding of polypeptides (Witkin et al., 1996). Moreover, it stimulates human sperm capacitation in the fallopian tube (Lachance et al., 2007). In human endometrium, HSP60 increased during the late proliferative and early secretory phase then decreased in the mid

to late secretory phase while other members of this family probably protect endometrial proteins against factors involvement in denaturant activity such as TNF-α, particularly in the implantation window (Tabibzadeh et al., 1999). Therefore, a significant reduction in HSP60 expression in CAR endometrium of the gravid horns during the early conceptus post-implantation period (present study) may be due to protein redundancy in which the other members of HSP family take over the role of HSP60 in the chaperon activity that is assumed to be required at the time of conceptus implantation.

A balance between protein synthesis and degradation of abnormal, damaged and short-lived proteins by proteasomes (Hochstrasser, 1995) is essential for several cellular processes, including cell cycle and division (King et al., 1996), proliferation and apoptosis (Naujokat and Hoffmann, 2002) and gene transcription (Muratani and Tansey, 2003). The turnover of proteins within cells by the ubiquitin-proteasome system depend on proteasome activators (Zhang et al., 1998). The proteasome activator 28 (PA28 or PSME) consists of two homologous subunits, PA28-alpha (or PSME1) and PA28-beta (or PSME2), each of which activates the proteasome (Zhang et al., 1998). Up-regulation of PA28-β protein expression observed in the present study in sheep CAR endometrium during the early conceptus post-implantion period had not been reported previuously. Of note is that PA28-beta protein expression increased dramatically in both the gravid and non gravid uterine horns suggesting a systemic rather than a local effect on endometrium PA28-β protein expression. These results suggest that factors present and associated with early pregnancy enhance PA28-β protein expression. The high level of PA28-β protein expression in CAR endometrium of the gravid and non gravid uterine horns (present study) likely suggests an important role for this regulated protein in protein-turnover since one can expect that the endometrium protein synthesis should be increased during the early post-implantation period. There should be evidence to suggest that proteasomes are parts of cellular defense mechanism against oxidative stress and protein oxidative damage by controlling the degradation of oxidatively damaged proteins (Ding et al. 2006; Poppek and Grune, 2006; Squier, 2006). Beside the high antioxidative capacity of the sheep CAR endometrium in the early conceptus post-implantation

period (Al-Gubory and Garrel, 2012), the dramatic post-implantation increase in PA28-β protein expression observed in the present study probably plays an important role in degradation of oxidised endometrium proteins during early pregnancy.

APOA1 is a main component of HDL synthesized by the liver and intestine (Zannis et al., 1985). In fertile women, APOA1 was down-regulated in secretory endometrium compared to proliferative endometrium (Brosens et al., 2010). In infertile women, Apo-A1 increased in mid-secretory phase endometrium as compared to early-secretory phase endometrium (Manohar et al., 2014). Deregulations of endometrial APOA1 protein (Fowler et al., 2007) and mRNA (Brosens et al., 2010) expression are important features of endometriosis in women. These findings suggest a role of Apo-A1 in endometrium preparation for conceptus implantation and development. HDL cholesterol and APOA1 play a crucial role in human embryo development (Baardman et al., 2013). Of note, the increased level of APOA1 secretion by blastocysts in spent media from cultures of high quality blastocysts compared to low quality blastocysts and this may be associated with implantation potential (Mains et al., 2011). Moreover, APOA1 is a source of nutrients for the early post-implanted conceptus (Assemat et al., 2005). In the present study, we showed that APOA1 was highly expressed in CAR endometrium from the gravid uterine horns at the early conceptus post-implantion period. Given APOA1 functions, it may be assumed that the implantaing conceptus exerts local effects on CAR areas of the sheep endometrium of the gravid horns to increase the production of apoA-I-containing lipoproteins necessary for early conceptus development and survival.

Transferrin (TF), an iron-binding and transport protein, is detected in sheep intrauterine luminal fluid between days 17 and 18 of pregnancy suggesting that TF is a conceptus-synthesized protein (Lee et al., 1998). However, it is unlikely that TF is synthesied and secreted solely by the developing conceptuses during the peri-implantatiuon periods. Indeed, it has been reported that porcine intrauterine fluid on day 16 of the oestous cycle or pregnancy contains high amount of TF (Vallet et al., 1996). In addition, TF protein expression in sheep CAR endometrium increased at

day 16 of the oestrous cycle as compared to the matching day of pregnancy (Al-Gubory et al., 2014). Interestingly, TF protein was highly expressed in CAR endometrium of the gravid and non gravid uterine horns on the day of conceptus attachment when compared with the early post-implantation period (present study). Therefore, under the physiologically relevant in vivo conditions of a unilaterally pregnant ewes and conceptus development, it is likely that the sheep endometrium is major source of TF during early pregnancy. Considering the role of TF in the proliferation and differentiation of mouse embryonic tissues in culture (Ekblom et al. 1981; Thesleff and Ekblom, 1985), it is likely that TF could be required for sheep conceptus develeopment during early pregnancy.

Galectins are a family of beta-galactoside-binding lectins. In the endometrial luminal epithelium of pregnant ewes, galectin-15 mRNA expression increased between days 12 and 16, and galectin-15 (LGALS15) protein in the uterine lumen increased between days 14 and 16 of pregnancy (Gray et al., 2004). LGALS15 is expressed uniquely in the endometrium of sheep and goats and plays an important role in trophoblast attachment (Lewis et al., 2007; Farmer et al., 2008). It is important to note that LGALS15 protein expression was not different between the gravid and non gravid uterine horns at the day of conceptus attachment and early post-implantation period (present study). Moreover, in the gravid uterine horns, LGALS15 decreased at post-implantation period when compared with the attachment day. These results suggest that the regulation of LGALS15 expression in sheep CAR endometrium likely does not depend on factors produced by the conceptus during early pregnancy.

In conclusion, our study provide evidence that conceptus-derived signals play key roles in the regulation of multiple functional proteins in sheep CAR endometrium, importantly AHCY, CA-II, HSP60 and APOA1 during conceptus implantation and the early post-implantation periods. These regulated proteins likely involved in providing a suitable intra-uterine environment required for conceptus attachment, implantation, early post-implantation development and successful establishment of pregnancy in sheep.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This project was funded by NHS Grampian R&D project number RG05/019.

Acknowledgements

We are grateful to M Fraser, I Davidson, E Stewart and E Argo for their expert technical assistance and to Dr P Cash for his proteomic advice (all IMS, University of Aberdeen). The authors thank the staff of the sheep sheds of Broueëssy and Jouy-en-Josas (INRA, France) for outstanding technical help and sheep management.

Author contributions

KHA jointly conceived and designed the study with PAF. KHA prepared the animal model, performed surgery and tissue collection. MA carried out the proteomic analysis, performed production and acquisition of data. KHA and MA wrote the manuscript. KHA and PAF contributed reagents and materials and helped in data interpretation. PAF made critical revisions of the manuscript for important intellectual content. All authors approved the final version of manuscript.

365 **Figure Legends**

366

367 **Figure 1.** Sheep caruncular endometrial proteome separated by 2DE gel using a 3-10 pH gradient.
368 A representative 2DE gel of the caruncle proteins from sheep non-gravid (NG) horn on day 20 of
369 pregnancy (NG20) is shown, indicating selected spots for cutting by arrows.

370

371 **Figure 2.** Expression changes of (A) adenosylhomocysteinase (AHCY), (B) carbonic anhydrase 2
372 (CA-II), and (C) heat shock 60kDa protein 1 (HSP60) in sheep caruncular endometrial tissues
373 collected from gravid horns (GH) and non-gravid horns (NG) of uteri at implantation (day 16 of
374 pregnancy) and post implantation (day 20 of pregnancy) periods. Normalised protein spot volumes
375 are shown as means ± SEM (n=4 ewes per group). Zoom boxes from the 2D gels showing the
376 identified proteins (arrows) are shown (right panels). The acceptable level of significance was set at
377 $P<0.05$.

378

379 **Figure 3.** Expression changes of (A) proteasome activator 28 beta (PA28beta), (B) apolipoprotein
380 A-1 (APOA1), (C), transferrin (TF) and (D) galectin 15 (LGALS15) in sheep caruncular
381 endometrial tissues collected from gravid horns (GH) and non-gravid horns (NG) of uteri at
382 implantation (day 16 of pregnancy) and post implantation (day 20 of pregnancy) periods.
383 Normalised protein spot volumes are shown as means ± SEM (n=4 ewes per group). Zoom boxes
384 from the 2D gels showing the identified proteins (arrows) are shown (right panels). The acceptable
385 level of significance was set at $P<0.05$.

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Table 1. Numbers of protein spots significantly (P<0.05) differing between caruncle of gravid horns (GH) and caruncle of non-gravid horns (NG) of sheep endometrium at the time of conceptus implantation (Day 16) and early post-implatation (Day 20) periods of pregnancy.

Groups compared					
Group	Compared with	Total number of spots	Up-regulated	Down-regulated	% of total spots
NG16	GH16	47	35	12	3
NG20	GH20	27	25	2	2
GH20	GH16	48	17	31	3
NG20	NG16	48	30	18	3

548 **Table 2** Caruncle proteins of gravid (GH) and non-gravid (NG) uterine horns demonstrating significant differences in expression at conceptus implantation
549 (Day 16) and early post-implantation (Day 20) periods of pregnancy. The significant fold changes are shown in bold with their corresponding P values
550 (P<0.05). Increases in spot volumes are denoted by a "+" and decreases by a "-" prefix to the fold-change values. The comparisons between groups follow the
551 rule that the fold-changes are calculated on the basis that the first group is being compared with the second group. Accession number is written regarding to
552 bovine species. The accession number specific for ovine protein is shown in brackets if available.

Protein	Spot no.	MW (KDa)	PI	MOWSE score (MASCOT)	Swiss-Prot	Fold change (P value)			
						NG16 vs. GH16	NG20 vs. GH20	GH20 vs. GH16	NG20 vs. NG16
Actin binding protein									
Gelsolin isoform b (GSN)	520	80.9	5.54	413	**Q3SX14**	+1.07	**+1.28 (0.031)**	-1.1	+1.08
Iron transport and homeostasis									
Transferrin (TF)	1463	79.8	6.75	473	**Q29443**	+1.04	+1.18	**-1.96 (0.001)**	**-1.73 (0.0001)**
Hydrolase									
Adenosylhomocysteinase (AdoHcyase) (AHCY)	964	48.1	5.88	932	**Q3MHL4**	**+1.27 (0.008)**	**+1.18 (0.03)**	-1.00	-1.08
Cytokine and nucleotide binding protein									
[p] High mobility group box 1 protein (HMGB1)	1242	25.0	5.75	416	**P63158**	**+1.32 (0.003)**	+1.07	+1.03	-1.20
[S] Cytokine induced protein 29 KDa (CIP29)		23.6	5.98	202	**Q2TBX1**				
Metalloenzyme									
Carbonic anhydrase 2 (CA-II)	1267	29.1	6.41	546	**P00922**	**-1.34 (0.0004)**	**-1.39 (0.049)**	+1.02	+1.06
Actin binding protein, heparin binding protein									
[p] Tropomyosin alpha-1 chain (TPM1)	1102	32.7	4.74	309	**Q91XN6**	-1.11	+1.06	+1.07	**+1.27 (0.013)**
[S] Hepatoma derived growth factor (HDGF)		26.3	4.84	176	**Q9XSK7**				

Chaperones									
Heat shock 60kDa protein 1 (HSP60)	791	61.1	5.71	1660	**P31081**	-1.02	**+1.27 (0.01)**	**-1.19 (0.044)**	+1.08
Amino acid metabolism, Metabolism									
[p] Glycine amidinotransferase, mitochondrial (GATM)	979	48.8	8	448	**Q2HJ74**	**+1.41 (0.04)**	+1.21	-1.12	-1.32
[S] Isocitrate dehydrogenase 1 (NADP+), soluble (IDH1)		47.1	6.34	365	**Q9XSG3**				
Cholesterol transport									
Apolipoprotein A-1 (APOA1)	1320	28.4	5.57	457	P02647	+1.11	-1.40	**+2.26 (0.01)**	+1.45
Protein degradation									
Proteasome activator subunit 2 (PA28beta) (PSME2)	1253	27.5	5.31	376	**Q5E9G3**	-1.12	+1.14	**+2.7 (0.0001)**	**+3.46 (0.0006)**
Ion transport									
Chloride intracellular channel protein 1 (**CLIC1**)	1231	23.8	5.12	169	**O00299**	-1.25	+1.07	+1.12	**+1.28 (0.03)**
Potassium channel tetramerisation domain containing 12 (KCTD12)	1109	47	5.68	260	616416 (NCBI)	-1.15	+1.04	+1.06	**+1.35 (0.0002)**
Cell adhesion									
Galectin 15 (LGALS15/OVGAL11)	1456	15.5	5.22	405	**Q19MU7***	-1.19	+1.52	**-1.91 (0.003)**	-1.05

* The accession number is for ovine species as the accession number for bovine was not found.

For 3 spots, peptide fragments were identified that belonged to more than one protein and the primary protein in the spot was identified based on 1) highest Mascot score, 2) best agreement between estimated (ie, from electrophoretic gel mobility) and calculated molecular weight and isoelectric point, and 3) highest peptide coverage.

[p] = primary protein in the spot ; [S] = secondary protein in the spot

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The sheep conceptus modulates proteome profiles in caruncular endometrium during early pregnancy

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Abbreviated title: Conceptus control of endometrium protein expression

Keywords: sheep, endometrium, proteome, gravid and non-gravid uterine horns, early pregnancy

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Abstract

The stage-specific expression of functional proteins within the endometrium, and their regulation by conceptus-derived signals, are crucial for conceptus development and successful establishment of pregnancy. Accurate knowledge of endometrium-conceptus interactions is key for the development of effective strategies to improve conceptus implantation rates both following natural conception and/or assisted reproductive technologies. The unilateral pregnant ewe provides a powerful experimental model for the study of endometrial function in the presence or absence of conceptuses during the peri-implantation period. Two-dimensional gel electrophoresis and mass spectrometry-based proteomics were used to compare and identify differentially expressed proteins in caruncular endometrium collected from the gravid uterine horns and the non-gravid uterine horns at the time of conceptus attachment (day 16 of pregnancy) and early post-implantation period (day 20 of pregnancy). Fifty seven protein spots were up-regulated in the gravid horn at day 16 of pregnancy and twenty seven protein spots were up-regulated in the gravid horn at day 20 of pregnancy. Sixteen proteins with different functions such as protein metabolism, cholesterol and ion transport and cell adhesion were identified. In conclusion, the use of the unilaterally pregnant ewe model provides evidence that the early implantation and post-implanting conceptus-derived signals up-regulate caruncle endometrial proteins, including carbonic anhydrase 2 (CA-II) and apolipoprotein A-1 (APOA1) and down-regulate caruncle endometrial proteins, including adenosylhomocysteinase (AHCY) and heat shock 60kDa protein 1 (HSP60). These regulated proteins are likely involved in providing a suitable intra-uterine environment required for conceptus attachment, implantation, early post-implantation development and the successful establishment of pregnancy in sheep.

Introduction

In sheep, goats and cattle, successful conceptus (embryo and associated extraembryonic membranes) implantation relies on elaborate cellular, biochemical and molecular cross-talk between the extraembryonic membranes and receptive uterine endometrial tissues that ensures corpus luteum (CL) progesterone production and optimal post-implantation conceptus development and survival (Paria et al., 2001; Imakawa et al., 2004). During early pregnancy, a high rate of embryonic morality occurs due to abnormal conceptus signalling (Goff, 2002; Dixon et al., 2007; Diskin and Morris, 2008). Although a multitude of molecular pathways involved in extraembryonic membrane-endometrium crosstalk during conceptus implantation and post-implantation periods have been identified through studies of gene expression, a comprehensive understanding of changes in many endometrium proteins expressed in the presence of conceptuses is currently lacking.

Our previous studies provided original evidence that several endometrial proteins with different functions, including protein synthesis and degradation, antioxidant defence, cell structural integrity, adhesion and signal transduction, play important roles in the establishment of early pregnancy in sheep (Al-Gubory et al., 2014). Of particular interest were proteins that were highly expressed in response to the presence of conceptuses at attachment and early post-implantation periods and, using our unilaterally pregnant ewe model, we demonstrated that the early implantation and post-implanting conceptus-derived signals up-regulate the expression of cytoplasmic tryptophanyl tRNA synthetase and the mitochondrial superoxide dismutase (Al-Gubory et al., 2014). The former is a key catalytic enzyme for the first step reaction in protein synthesis (Sallafranque et al., 1986) and the latter the first antioxidant defence enzyme against reactive oxygen species-induced mitochondrial oxidative damage (Orrenius et al., 2007), in sheep caruncular endometrium (Al-Gubory et al., 2015). In sheep, the endometrium caruncles (CAR) are highly vascularized stromal protuberances covered by a simple luminal epithelium. CAR areas are specialized sites of attachment of the outer covering extraembryonic membrane, the trophectoderm, and are privileged

endometrial tissues for conceptus-uterine communication. Our hypothesis is that the early developing sheep conceptus modulates protein expression profiles in CAR endometrium during early pregnancy.

The unilateral pregnant sheep model enables changes in the expression of endometrial proteins in the presence or absence of conceptuses to be studied, providing a powerful model for the investigation of proteome changes during the peri-implantation period (Al-Gubory et al., 2015). The benefit of this model is that both uterine horns are exposed to similar concentrations of circulating hormones such as progesterone but only the gravid horn is under the direct action of local signalling molecules produced by the conceptus. In the present study, the unilateral pregnant ewes with functional ovaries were therefore employed to test our hypothesis. Two-dimensional gel electrophoresis (2DE) based proteomics (Fowler et al., 2007; Arianmanesh et al., 2011; Al-Gubory et al., 2014) was used to characterize specific alterations in the proteome of CAR endometrial tissues collected from the gravid uterine horns (GH) and the non-gravid uterine horns (NG) at the time of conceptus attachment (day 16 of pregnancy) and early post-implantation period (day 20 of pregnancy).

Materials and Methods

Experimental animals

All procedures relating to care and use of animals were approved by the French Ministry of Agriculture according to the French regulation for animal experimentation (authorization no° 78-34). Ewes of the Préalpes-du-Sud breed (18 months of age) were used in this study. Unilaterally pregnant ewes were prepared surgically as described previously (Payne and Lamming, 1994; Lamming et al., 1995). Briefly, ewes were initially anesthetized with a mixture of pentobarbital (Sanofi, Paris, France) and thiopentone (Abbott, Aubervilliers, France). After endotracheal intubation, general anaesthesia was maintained by constant inhalation of a mixture of oxygen and halothane. Reproductive organs were exposed via midventral laparotomy and one ovary was removed. One uterine horn was ligated close to the uterine bifurcation so that after mating the

conceptus is confined to the non-ligated pregnant horn. Three weeks after surgery, the ewes were treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France) to synchronize oestrous. Ewes were mated twice with fertile rams of the same breed, at an interval of 12 h during the synchronized oestrus. The ewes were housed under conditions of natural day-length and temperature and had free access to mineral licks and water.

Endometrial tissue collection

The ewes were slaughtered at a local abattoir in accordance with protocols approved by the local institutional animal use committee at the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France). Pregnant ewes were randomly allocated for slaughter at two specific stages of early pregnancy corresponding to the initial conceptus attachment to CAR areas (day 16, n=4 ewes) and the early conceptus post-implantation period (day 20, n=4 ewes). The stages of pregnancy were confirmed by the presence and the morphology of the conceptus in uterine flushings. Immediately after slaughter of the ewes, the reproductive tracts were collected, placed on crushed ice and transported to the laboratory. Endometrial CAR areas were collected from the entire GH (ipsilateral uterine horn) and NG (contralateral uterine horn) of each ewe, snap-frozen in liquid nitrogen and stored at -80 °C until processed for 2DE gel electrophoresis and Western blot.

Protein extraction and quantification for electrophoretic analysis

CAR from the gravid horns on days 16 (GH16) and 20 (GH20), non-gravid horns on days 16 (NG16) and 20 (NG20) were processed separately for 1DE and 2DE gel electrophoresis as described previously (Fowler et al., 2007). Briefly, tissues were combined with 5 ml lysis buffer/1 mg wet weight of tissue. The lysis buffer (0.01 M Tris–HCl, pH 7.4) contained 1 mM EDTA, 8 M urea, 0.05 M dithiothreitol, 10% (v/v) glycerol 5% (v/v), NP40, 6% (w/v), pH 3–10, resolyte (Merck Eurolab Ltd, Poole, Dorset, UK) and protease inhibitor cocktail (Roche Diagnostics). The tissues were disrupted using a Tissue Lyser (Qiagen Ltd) for 4 min at 30 Hz. Insoluble materials were removed from the lysates by centrifugation (50,000 g at 4°C) for 30 min. The protein content

of the final supernatant had been determined by RC-DC assay (Bio-Rad Laboratories Ltd). The protein extracts were stored at -80°C until required for further analysis.

Two dimensional gel electrophoresis (2DE) analysis

Equal amounts of protein from CAR of each ewe in each group were combined to make 4 protein pools (800 μg protein in each pool): gravid horn on days 16 (GH16) and 20 (GH20), non-gravid horn on days 16 (NG16) and 20 (NG20). 2DE was performed as described (Cash et al., 2003). As a first dimension separation, 70 μg of total protein from each pool was loaded onto 7 cm immobiline™ DryStrip non-linear pH gradient (IPG) strips of pH 3-10 (GE Healthcare, UK). The second dimension was carried out using 13 cm NUPAGE® Novex 4-12%, Bis-Tris Zoom® gels (Invitrogen Ltd, Paisley, UK). Quadruplicate 2DE gels were prepared for each of the 4 groups (representative gel is shown in Figure 1). Proteins were visualized using Colloidal CBB G-250 and scanned using an ImageScanner™ III (GE Healthcare). Protein spot profiles were analysed using Progenesis SameSpots version 3 software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) as described (Arianmanesh et al., 2011). Briefly, reference gel was selected and the other gels were aligned to be closely matched to this reference gel. Background was subtracted individually from each gel and spot volumes were normalised relative to total spot volume individually for each gel. Ultimately, 15 were selected for identification by LC-MS/MS on the basis of significance (log-normalised spot volumes had to differ between two groups at the level of $P<0.05$ by ANOVA and post-hoc testing), spot volume (a difference of a $\geq$1.25-fold increase or decrease between two groups), concentrating on the most abundant proteins with the most stable expression across the 4 replicate gels for each group.

Mass spectrometry

To identify proteins, 15 selected spots were excised from stained gels and subjected to in-gel trypsin digestion as described previously (Uwins et al. 2006). The peptide fragment mass spectra

were acquired on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer operated in the reflection delayed extraction mode. Tryptic peptides from the MS/MS spectra were applied to search the NCBI (National Centre for Biotechnology Information) database with the MASCOT program (http://www.matrixscience.com). Search parameters for the programme included maximum allowed error of peptide mass 250 ppm, cysteine as S-carbamidomethyl-derivative and oxidation of methionine were allowed.

Statistical Analysis

Normality of data was tested with the Shapiro-Wilk test. Normally distributed data were subjected to one- and two-way ANOVA and Bonferroni post-hoc test using SPSS 17.0 software to assess significance of differences. Statistical comparisons between specific groups were carried out by student's t-test. Differences were considered significant at $P<0.05$.

Results

Overall, 998 protein spots were included (on the basis of clear, reproducible expression and absence of noise in all four gels for each group) for analysis from a total of 1482 distinct protein spots detected by automatic detection with Progenesis SameSpots Software. The number of spots showing statistically significant differences in normalized spot volumes between groups is shown in Table 1.

Comparison between the GH and the NG uterine horns at day 16 of pregnancy revealed that 47 (3%) of protein spots were significantly changed ($P<0.05$). Among these, 35 normalized spot volumes were up-regulated and 12 normalized spot volumes were down-regulated (Table 1). Comparison between the GH and the NG uterine horns at day 20 of pregnancy revealed that 27 (2%) of protein spots were significantly changed ($P<0.05$). 25 of these normalized spot volumes were up-regulated and 2 normalized spot volumes were down-regulated (Table 1). In GH uterine horns, 48 (3%) of protein spots were significantly changed ($P<0.05$) between days 16 and 20 of

pregnancy. In this category, 17 normalized spot volumes were up-regulated and 31 normalized spot volumes were down-regulated (Table 1). In NG uterine horns, 48 (3%) of protein spots were significantly changed ($P<0.05$) between days 16 and 20 of pregnancy. Among these, 30 normalized spot volumes were up-regulated and 18 normalized spot volumes were down-regulated (Table 1).

The proteins spots in GH and NG uterine horns exhibiting significant differences in expression at implantation day and early post-implantation period and identified are shown in Table 2. Adenosylhomocysteinase (AHCY, Figure 2A) increased ($P<0.05$) in NG uterine horns compared to GH uterine horns at both days 16 and 20 of pregnancy. (Table 2). Carbonic anhydrase 2 (CA-II, Figure 2B), increased ($P<0.05$) in GH uterine horns compared to NG uterine horns at both days 16 and 20 of pregnancy (Table 2). Heat shock 60 kDa protein 1 (HSP60, Figure 2C), increased ($P<0.05$) in NG uterine horns compared to GH uterine horns at day 20 of pregnancy. (Table 2). In GH uterine horns, HSP60 decreased (Figure 2C, $P<0.05$) at day 20 when compared with day 16 of pregnancy (Table 2).

In GH and NG uterine horns, proteasome activator subunit 2 (PA28beta/PSME2, Figure 3A) increased ($P<0.05$) at day 20 when compared with day 16 of pregnancy (Table 2). In GH uterine horns, apolipoprotein A-1 (APOA1, Figure 3B) increased ($P<0.05$) at day 20 when compared with day 16 of pregnancy (Table 2). In GH and NG uterine horns, transferrin (TF, Figure 3C) decreased ($P<0.05$) at day 20 when compared with the day 16 of pregnancy (Table 2). In GH uterine horns, galectin 15 (LGALS15, Figure 3D) decreased ($P<0.05$) at day 20 when compared with day 16 of pregnancy (Table 2).

Discussion

The establishment of pregnancy requires correctly timed, exquisitely coordinated, adaptive, responses of the uterine endometrium to the presence of conceptuses. Some of these responses will be via modification of the expression of functional proteins during early pregnancy. The proteomic profile of sheep CAR endometrium reported here provided a new understanding about the role of

conceptus-derived signals in the regulation of a substantial number of functional proteins involved in iron transport and homeostasis, hydrolysis, protein chaperoning and degradation, amino acid metabolism, cholesterol transport and cell adhesion.

Conceptus-derived factors reduce the expression of AHCY protein as evidenced by the down-regulation of this protein in endometrial caruncular tissues of the gravid uterine horns compared with the non-gravid uterine horns at both implantation and post-implantation periods (present study). AHCY catalyzes the breakdown of S-adenosylhomocysteine (AdoHcy) to adenosine (Ado) and L-homocysteine (Hcy) (Turner et al., 2000). It is imporatnt to note that hyperhomocysteinemia (HHcy) exerts adverse effects through the induction of inflammation pathways, including endothelial monocyte adhesion and infiltration (Wang et al., 2002), oxidative stress, activation of pro-inflammatory factors and endothelial dysfunction (Lawrence de Koning et al. 2003). Hcy activates NADPH oxidase and increases reactive oxygen species in human umbilical vein endothelial cells (Dong et al., 2005). Elevated level of Hcy within organs and tissues is therefore a potentially pathophysiological risk factor for uterine endothelial function via an enhancement of oxidative stress and inflammation. Maternal Hhcy shoud be associated with placental abruption and spontaneous abortion (Ray and Laskin, 1999). Increased Hcy levels and oxidative stress represent a risk factor for the establishment and maintenance of pregnancy (Micle et al. 2012). Therefore, the conceptus must hold Hcy in check within CAR endometrium at conceptus attachment (Day 16) and early post-implantation period (Day 20) of pregnancy through down-regulation of AHCY protein expression (present study). We suggest that the conceptus-derived factors exert local effects within the endometrium to counteract peri-implantation oxidative stress through the control of Hcy production and thereby support the establishment of pregnancy.

Upregulation of CA-II protein expression by conceptus-derived signals observed here on the day of conceptus implantation and during early post-implantation period had not been reported previously in endometrium of any mammalian species. Identification of these signaling molecules is essential in our understanding of the molecular mechanisms that should be involved in the establishment of

pregnancy. CAII catalyzes the reversible hydration of carbon dioxide to bicarbonate and plays an important role in acid-base homeostasis within tissues of biological systems (Khalifah, 1971). These reactions are requisite for cancer development, invasion and progression. Interestingly, CA II is highly expressed in tumours of different organs, including brain (Parkkila et al., 1995a), pancreas (Parkkila et al., 1995b) and kidney (Parkkila et al., 2000), where it favourably induces an environment necessary for the growth and spread of the tumour by changing acidity of the extracellular medium surrounding cancer cells. In the neonatal mouse uterus, where members of the CA family are expressed (Hu et al., 2004), CAII mRNAs were localized in epithelial and stromal cells of the endometrium suggesting a functional role for CAII in endometrial gland development during postnatal uterine development (Hu and Spencer, 2005). The expression of CA II in the bovine (Nishita et al., 1990) and human (Aliakbar et al., 1990; Muhlhauser et al., 1994) placentas supports the suggestion that this enzyme is required for endometrial tissue remodelling. Endometrium structural remodelling in ruminants, including sheep, plays crucial role in implantation, placentation and conceptus nutrition (Igwebuike, 2009). On the day of conceptus attachment (day 16 of pregnancy), there is close contact between trophoblast, the extra-embryonic membrane of the conceptus, and the epithelium overlying CAR endometrium, over raised areas of the endometrium, to allow implantation and early placental development. The high level of CA-II protein expression in CAR endometrium of the gravid uterine horns (present study) likely suggests an important role for this regulated protein in promoting trophoblast attachment, invasion and fusion with endometrial epithelium and/or remodelling the endometrium for successful early conceptus implantation and, consequently, formation of the maternal-fetal interface during placental development.

HSP60 has multiple functions in the normal cells, including inter-organelle transport, inhibition of aggregation of denatured polypeptides, antigen presentation, pro-apoptotic activity (Yu et al., 2012) and promotion of the proper folding of polypeptides (Witkin et al., 1996). Moreover, it stimulates human sperm capacitation in the fallopian tube (Lachance et al., 2007). In human endometrium, HSP60 increased during the late proliferative and early secretory phase then decreased in the mid

263 to late secretory phase while other members of this family probably protect endometrial proteins
264 against factors involvement in denaturant activity such as TNF-α, particularly in the implantation
265 window (Tabibzadeh et al., 1999). Therefore, a significant reduction in HSP60 expression in CAR
266 endometrium of the gravid horns during the early conceptus post-implantation period (present
267 study) may be due to protein redundancy in which the other members of HSP family take over the
268 role of HSP60 in the chaperon activity that is assumed to be required at the time of conceptus
269 implantation.
270
271 A balance between protein synthesis and degradation of abnormal, damaged and short-lived
272 proteins by proteasomes (Hochstrasser, 1995) is essential for several cellular processes, including
273 cell cycle and division (King et al., 1996), proliferation and apoptosis (Naujokat and Hoffmann,
274 2002) and gene transcription (Muratani and Tansey, 2003). The turnover of proteins within cells by
275 the ubiquitin-proteasome system depend on proteasome activators (Zhang et al., 1998). The
276 proteasome activator 28 (PA28 or PSME) consists of two homologous subunits, PA28-alpha (or
277 PSME1) and PA28-beta (or PSME2), each of which activates the proteasome (Zhang et al., 1998).
278 Up-regulation of PA28-β protein expression observed in the present study in sheep CAR
279 endometrium during the early conceptus post-implantion period had not been reported previuously.
280 Of note is that PA28-beta protein expression increased dramatically in both the gravid and non
281 gravid uterine horns suggesting a systemic rather than a local effect on endometrium PA28-β
282 protein expression. These results suggest that factors present and associated with early pregnancy
283 enhance PA28-β protein expression. The high level of PA28-β protein expression in CAR
284 endometrium of the gravid and non gravid uterine horns (present study) likely suggests an
285 important role for this regulated protein in protein-turnover since one can expect that the
286 endometrium protein synthesis should be increased during the early post-implantation period.
287 There should be evidence to suggest that proteasomes are parts of cellular defense mechanism
288 against oxidative stress and protein oxidative damage by controlling the degradation of oxidatively
289 damaged proteins (Ding et al. 2006; Poppek and Grune, 2006; Squier, 2006). Beside the high
290 antioxidative capacity of the sheep CAR endometrium in the early conceptus post-implantation

period (Al-Gubory and Garrel, 2012), the dramatic post-implantation increase in PA28-β protein expression observed in the present study probably plays an important role in degradation of oxidised endometrium proteins during early pregnancy.

APOA1 is a main component of HDL synthesized by the liver and intestine (Zannis et al., 1985). In fertile women, APOA1 was down-regulated in secretory endometrium compared to proliferative endometrium (Brosens et al., 2010). In infertile women, Apo-A1 increased in mid-secretory phase endometrium as compared to early-secretory phase endometrium (Manohar et al., 2014). Deregulations of endometrial APOA1 protein (Fowler et al., 2007) and mRNA (Brosens et al., 2010) expression are important features of endometriosis in women. These findings suggest a role of Apo-A1 in endometrium preparation for conceptus implantation and development. HDL cholesterol and APOA1 play a crucial role in human embryo development (Baardman et al., 2013). Of note, the increased level of APOA1 secretion by blastocysts in spent media from cultures of high quality blastocysts compared to low quality blastocysts and this may be associated with implantation potential (Mains et al., 2011). Moreover, APOA1 is a source of nutrients for the early post-implanted conceptus (Assemat et al., 2005). In the present study, we showed that APOA1 was highly expressed in CAR endometrium from the gravid uterine horns at the early conceptus post-implantion period. Given APOA1 functions, it may be assumed that the implantaing conceptus exerts local effects on CAR areas of the sheep endometrium of the gravid horns to increase the production of apoA-I-containing lipoproteins necessary for early conceptus development and survival.

Transferrin (TF), an iron-binding and transport protein, is detected in sheep intrauterine luminal fluid between days 17 and 18 of pregnancy suggesting that TF is a conceptus-synthesized protein (Lee et al., 1998). However, it is unlikely that TF is synthesied and secreted solely by the developing conceptuses during the peri-implantatiuon periods. Indeed, it has been reported that porcine intrauterine fluid on day 16 of the oestous cycle or pregnancy contains high amount of TF (Vallet et al., 1996). In addition, TF protein expression in sheep CAR endometrium increased at

day 16 of the oestrous cycle as compared to the matching day of pregnancy (Al-Gubory et al., 2014). Interestingly, TF protein was highly expressed in CAR endometrium of the gravid and non gravid uterine horns on the day of conceptus attachment when compared with the early post-implantation period (present study). Therefore, under the physiologically relevant in vivo conditions of a unilaterally pregnant ewes and conceptus development, it is likely that the sheep endometrium is major source of TF during early pregnancy. Considering the role of TF in the proliferation and differentiation of mouse embryonic tissues in culture (Ekblom et al. 1981; Thesleff and Ekblom, 1985), it is likely that TF could be required for sheep conceptus develeopment during early pregnancy.

Galectins are a family of beta-galactoside-binding lectins. In the endometrial luminal epithelium of pregnant ewes, galectin-15 mRNA expression increased between days 12 and 16, and galectin-15 (LGALS15) protein in the uterine lumen increased between days 14 and 16 of pregnancy (Gray et al., 2004). LGALS15 is expressed uniquely in the endometrium of sheep and goats and plays an important role in trophoblast attachment (Lewis et al., 2007; Farmer et al., 2008). It is important to note that LGALS15 protein expression was not different between the gravid and non gravid uterine horns at the day of conceptus attachment and early post-implantation period (present study). Moreover, in the gravid uterine horns, LGALS15 decreased at post-implantation period when compared with the attachment day. These results suggest that the regulation of LGALS15 expression in sheep CAR endometrium likely does not depend on factors produced by the conceptus during early pregnancy.

In conclusion, our study provide evidence that conceptus-derived signals play key roles in the regulation of multiple functional proteins in sheep CAR endometrium, importantly AHCY, CA-II, HSP60 and APOA1 during conceptus implantation and the early post-implantation periods. These regulated proteins likely involved in providing a suitable intra-uterine environment required for conceptus attachment, implantation, early post-implantation development and successful establishment of pregnancy in sheep.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This project was funded by NHS Grampian R&D project number RG05/019.

Acknowledgements

We are grateful to M Fraser, I Davidson, E Stewart and E Argo for their expert technical assistance and to Dr P Cash for his proteomic advice (all IMS, University of Aberdeen). The authors thank the staff of the sheep sheds of Broueëssy and Jouy-en-Josas (INRA, France) for outstanding technical help and sheep management.

Author contributions

KHA jointly conceived and designed the study with PAF. KHA prepared the animal model, performed surgery and tissue collection. MA carried out the proteomic analysis, performed production and acquisition of data. KHA and MA wrote the manuscript. KHA and PAF contributed reagents and materials and helped in data interpretation. PAF made critical revisions of the manuscript for important intellectual content. All authors approved the final version of manuscript.

Figure Legends

Figure 1. Sheep caruncular endometrial proteome separated by 2DE gel using a 3-10 pH gradient. A representative 2DE gel of the caruncle proteins from sheep non-gravid (NG) horn on day 20 of pregnancy (NG20) is shown, indicating selected spots for cutting by arrows.

Figure 2. Expression changes of (A) adenosylhomocysteinase (AHCY), (B) carbonic anhydrase 2 (CA-II), and (C) heat shock 60kDa protein 1 (HSP60) in sheep caruncular endometrial tissues collected from gravid horns (GH) and non-gravid horns (NG) of uteri at implantation (day 16 of pregnancy) and post implantation (day 20 of pregnancy) periods. Normalised protein spot volumes are shown as means ± SEM (n=4 ewes per group). Zoom boxes from the 2D gels showing the identified proteins (arrows) are shown (right panels). The acceptable level of significance was set at $P<0.05$.

Figure 3. Expression changes of (A) proteasome activator 28 beta (PA28beta), (B) apolipoprotein A-1 (APOA1), (C), transferrin (TF) and (D) galectin 15 (LGALS15) in sheep caruncular endometrial tissues collected from gravid horns (GH) and non-gravid horns (NG) of uteri at implantation (day 16 of pregnancy) and post implantation (day 20 of pregnancy) periods. Normalised protein spot volumes are shown as means ± SEM (n=4 ewes per group). Zoom boxes from the 2D gels showing the identified proteins (arrows) are shown (right panels). The acceptable level of significance was set at $P<0.05$.

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535

Table 1. Numbers of protein spots significantly (P<0.05) differing between caruncle of gravid horns (GH) and caruncle of non-gravid horns (NG) of sheep endometrium at the time of conceptus implantation (Day 16) and early post-implatation (Day 20) periods of pregnancy.

Groups compared					
Group	Compared with	Total number of spots	Up-regulated	Down-regulated	% of total spots
NG16	GH16	47	35	12	3
NG20	GH20	27	25	2	2
GH20	GH16	48	17	31	3
NG20	NG16	48	30	18	3

548 **Table 2** Caruncle proteins of gravid (GH) and non-gravid (NG) uterine horns demonstrating significant differences in expression at conceptus implantation
549 (Day 16) and early post-implantation (Day 20) periods of pregnancy. The significant fold changes are shown in bold with their corresponding P values
550 (P<0.05). Increases in spot volumes are denoted by a "+" and decreases by a "-" prefix to the fold-change values. The comparisons between groups follow the
551 rule that the fold-changes are calculated on the basis that the first group is being compared with the second group. Accession number is written regarding to
552 bovine species. The accession number specific for ovine protein is shown in brackets if available.

Protein	Spot no.	MW (KDa)	PI	MOWSE score (MASCOT)	Swiss-Prot	Fold change (P value)			
						NG16 vs. GH16	NG20 vs. GH20	GH20 vs. GH16	NG20 vs. NG16
Actin binding protein									
Gelsolin isoform b (GSN)	520	80.9	5.54	413	**Q3SX14**	+1.07	**+1.28 (0.031)**	-1.1	+1.08
Iron transport and homeostasis									
Transferrin (TF)	1463	79.8	6.75	473	**Q29443**	+1.04	+1.18	**-1.96 (0.001)**	**-1.73 (0.0001)**
Hydrolase									
Adenosylhomocysteinase (AdoHcyase) (AHCY)	964	48.1	5.88	932	**Q3MHL4**	**+1.27 (0.008)**	**+1.18 (0.03)**	-1.00	-1.08
Cytokine and nucleotide binding protein									
[p] High mobility group box 1 protein (HMGB1)	1242	25.0	5.75	416	**P63158**	**+1.32 (0.003)**	+1.07	+1.03	-1.20
[S] Cytokine induced protein 29 KDa (CIP29)		23.6	5.98	202	**Q2TBX1**				
Metalloenzyme									
Carbonic anhydrase 2 (CA-II)	1267	29.1	6.41	546	**P00922**	**-1.34 (0.0004)**	**-1.39 (0.049)**	+1.02	+1.06
Actin binding protein, heparin binding protein									
[p] Tropomyosin alpha-1 chain (TPM1)	1102	32.7	4.74	309	**Q91XN6**	-1.11	+1.06	+1.07	**+1.27 (0.013)**
[S] Hepatoma derived growth factor (HDGF)		26.3	4.84	176	**Q9XSK7**				

Chaperones									
Heat shock 60kDa protein 1 (HSP60)	791	61.1	5.71	1660	**P31081**	-1.02	**+1.27 (0.01)**	**-1.19 (0.044)**	+1.08
Amino acid metabolism, Metabolism									
[p] Glycine amidinotransferase, mitochondrial (GATM)	979	48.8	8	448	**Q2HJ74**	**+1.41 (0.04)**	+1.21	-1.12	-1.32
[S] Isocitrate dehydrogenase 1 (NADP+), soluble (IDH1)		47.1	6.34	365	**Q9XSG3**				
Cholesterol transport									
Apolipoprotein A-1 (APOA1)	1320	28.4	5.57	457	P02647	+1.11	-1.40	**+2.26 (0.01)**	+1.45
Protein degradation									
Proteasome activator subunit 2 (PA28beta) (PSME2)	1253	27.5	5.31	376	**Q5E9G3**	-1.12	+1.14	**+2.7 (0.0001)**	**+3.46 (0.0006)**
Ion transport									
Chloride intracellular channel protein 1 (**CLIC1**)	1231	23.8	5.12	169	**O00299**	-1.25	+1.07	+1.12	**+1.28 (0.03)**
Potassium channel tetramerisation domain containing 12 (KCTD12)	1109	47	5.68	260	616416 (NCBI)	-1.15	+1.04	+1.06	**+1.35 (0.0002)**
Cell adhesion									
Galectin 15 (LGALS15/OVGAL11)	1456	15.5	5.22	405	**Q19MU7***	-1.19	+1.52	**-1.91 (0.003)**	-1.05

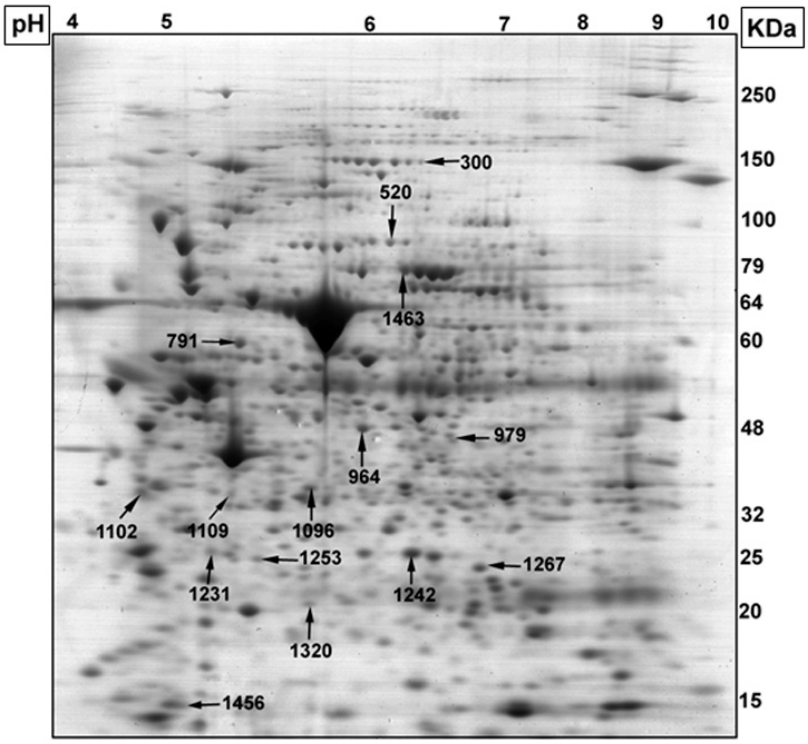
* The accession number is for ovine species as the accession number for bovine was not found.

For 3 spots, peptide fragments were identified that belonged to more than one protein and the primary protein in the spot was identified based on 1) highest Mascot score, 2) best agreement between estimated (ie, from electrophoretic gel mobility) and calculated molecular weight and isoelectric point, and 3) highest peptide coverage.

[p] = primary protein in the spot ; [S] = secondary protein in the spot

559

Figure 1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7
8
9
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KDa
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150
100
79
64
60
48
32
25
20
15
300
520
1463
791
979
964
1102
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1096
1253
1267
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1242
1320
1456

Figure 2

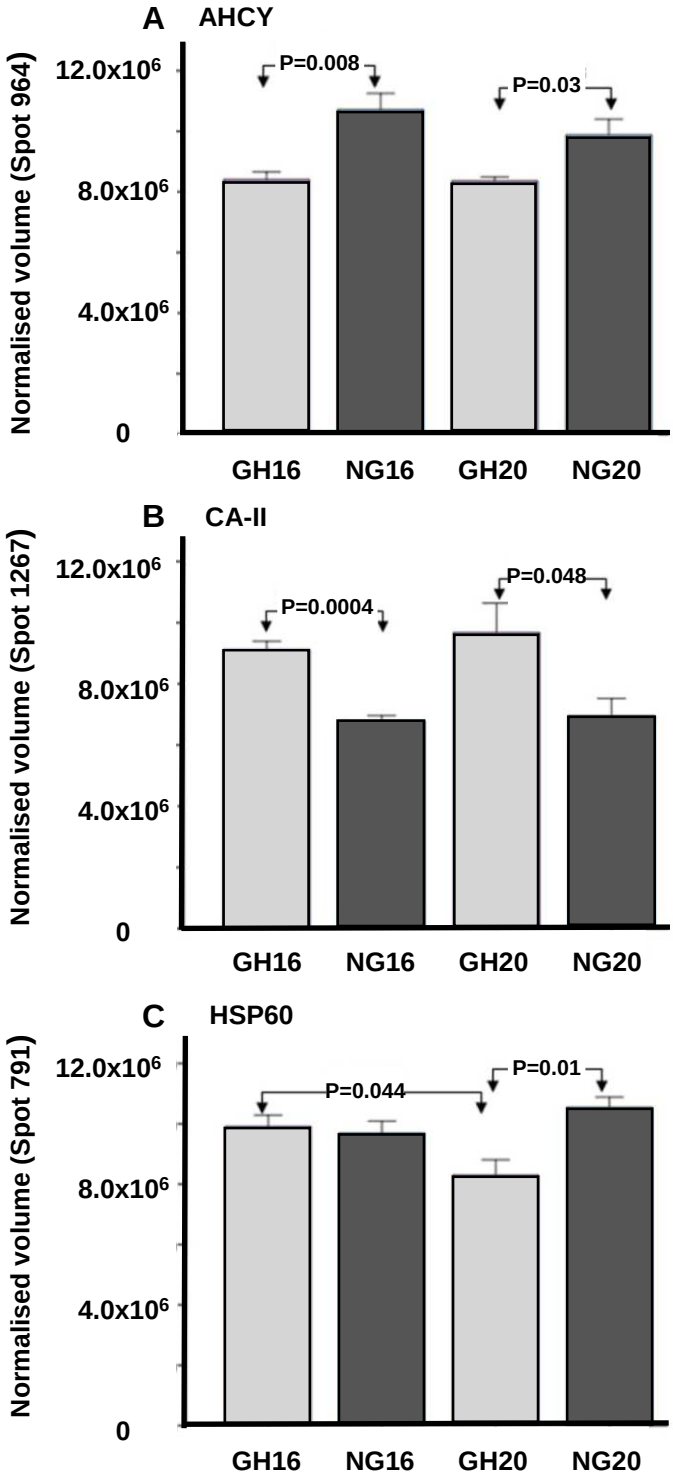
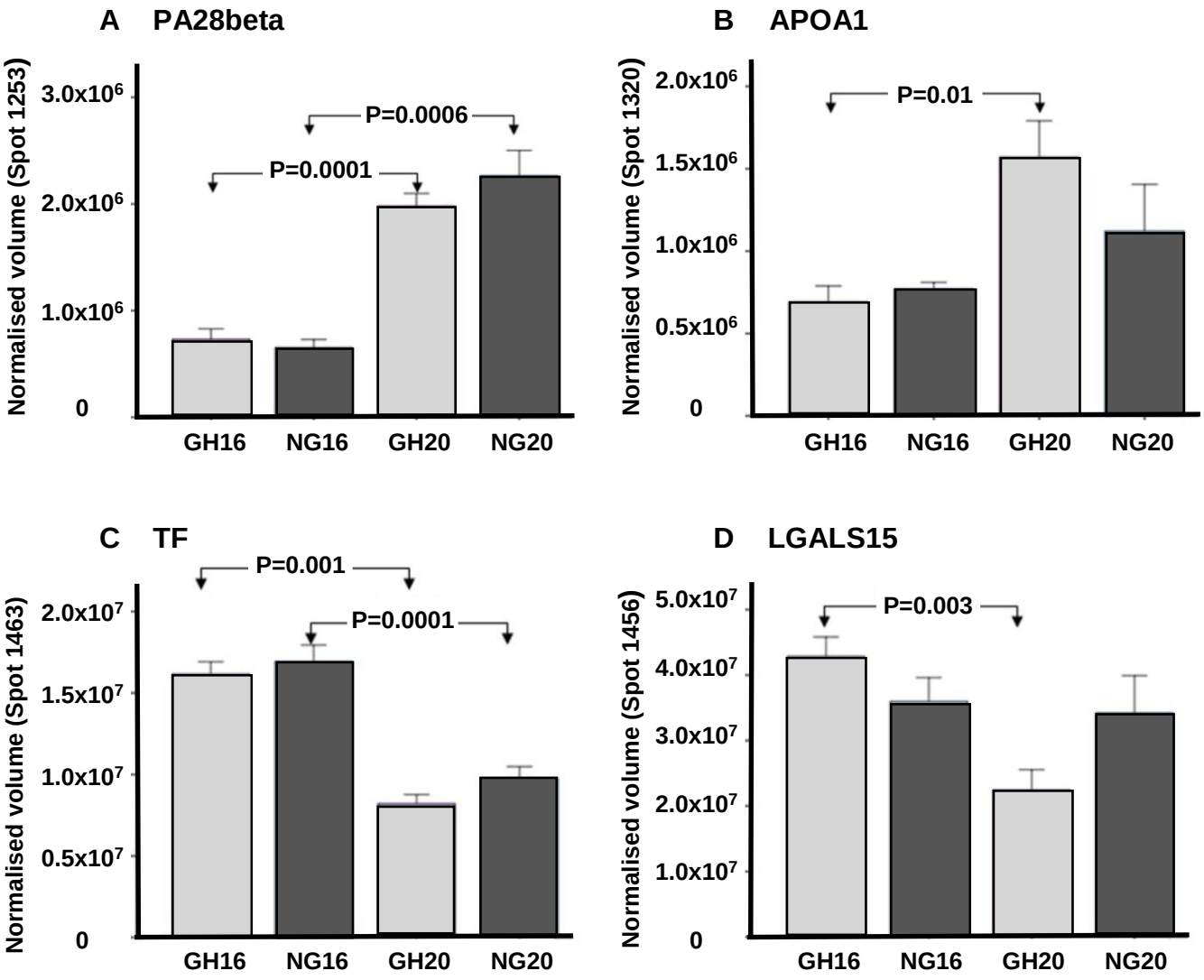


Figure 3



Conflict of Interest Statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.